Undergraduate Research Thesis

Agonist peptide inhibition by antagonist peptide during T cell activation: global or local effect on T cell

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Abstract

T-cell activation is one of the key mechanisms for the generation of adaptive immunity. This process begins from the T-cell receptor (TCR) recognition of agonist ligand presented by antigen presenting cells (APC) like dendritic cells. It is known that multiple interactions between agonist ligand and TCR are required to activate a T-cell, while similar interactions between antagonist ligand and TCR would only weakly activate the T-cell. Although much is known about T-cell activation due to agonist and antagonist ligands, few experiments in the past have shown how T-cells react to both antagonist and agonist ligands at the same time; which is shown to be the case during several infections. Using a micropipette adhesion frequency assay multiple scenarios involving agonist and antagonist ligands can be simulated. Interactions that were looked at involved spatial and time differences between agonist and antagonist stimulation of the T-cell. For each type of ligand stimulation responses from at least three T cells were recorded. A one-way ANOVA or student's t-test was used for analysis of the data for each stimulation type. Results from the experiments using local with simultaneous simulation and global stimulation proved to be significant when agonist stimulation only and agonist plus antagonist stimulation were compared, however the results from local and time delayed experiment proved to be insignificant. Both local with simultaneous presentation and global stimulation showed how the addition of antagonist stimulation can cause the T-cell exhibit a higher adhesion frequency to agonist ligand, especially with contact times under one second. When we planned those experiments we expected that antagonist ligands inhibition of agonist stimulation would be seen as effect opposite to the one observed. We expected to see the lowering of the adhesion frequency . However, local and time-delay stimulation showed a more complex behavior of T-cells in their reaction to stimulus. In the future with knowledge of these interactions it will be possible to focus in on the exact mechanism of T-cell activation modulation by antagonist ligands.

Introduction

The purpose of my research at Dr. Zhu's Cellular and Molecular Biomechanics Laboratory at Georgia Institute of Technology was to look into how antagonist and agonist peptides complexed with major histocombatibility complexes (pMHCs) interacted with the T-cell to inhibit or expedite its activation. A T-cell can be activated by the presentation of agonist pMHC on the surface of an antigen presenting cell (APC) binding to the T-cell receptor complex (TCR) a number of times great enough to initiate intra cellular signaling. The amount of pMHCs needed to activate a T-cell depends on the quality of the peptide and its affinity for TCR. Despite the fact that only agonist pMHC is needed to activate the T-cell, in normal biological scenarios agonist pMHCs are not the only pMHCS present on the APC. Antagonist pMHCS, ones that bind to a receptor cause weak modified cellular response, are often present in the body during infection. However, there have been few studies that look at how T-cells react to combined stimulus of these two types of pMHCs; agonist and antagonist.

By using adhesion frequency assays between red blood cells (RBCs) and T-cells it is possible to determine the extent of T-cell recognition of surface ligands coated on the red blood cell. Through a specific protocol of stimulation by both antagonist and agonist ligands the goal is to show how antagonist ligands affect T-cell recognition of agonist ligands, and thusly T-cell activation. To determine the global effect of antagonist ligands on T-cell activation, microbeads coated in antagonist ligand were presented to the T-cell at the same time as the RBC coated in agonist ligand, however in a different location on the T-cell. For determining the local effect on activation, first the microbead with antagonist were presented for 1 minute then the RBC with agonist were presented directly after antagonist stimulation in the same spot and adhesion frequency has be taken before and after stimulation with antagonist.

Methods

Preparation of Red Blood Cells and pMHCs

RBCs were isolated from whole blood of healthy volunteers according to protocols approved by the Institutional Review Board of Georgia Institute of Technology. Naive CD8 OT1 T cells were generated according to Emory University IACUC approved protocols. Ovalbumin-derived peptides OVA, E1, and R4, and a vesicular stomatitis virus-derived peptide VSV were synthesized using a standard F-moc (9-fluorenylmethyloxycarbonyl) chemistry on a Symphony/Multiplex Peptide Synthesizer and analyzed by HPLC and mass spectrometry at the Chemistry Core Facility of the Department of Microbiology and Immunology, Emory University School of Medicine. Peptides bound to monomeric wild-type H-2Kb tagged with a single biotin at the C terminus were produced by the NIH Tetramer Core Facility at Emory University. pMHCs were stored at -80 C and a fresh aliquot was used in each experiment. To couple pMHC onto RBCs and beads monomeric pMHCs were coated onto RBCs or polystyrene beads by biotin–streptavidin coupling. RBCs were biotinylated using different concentrations (to vary the pMHC density) of biotin-X-NHS (Calbiochem) per manufacturer's instruction incubated with either 1mg/ml tetrameric wide-type streptavidin (Pierce) for 30 min at 4 C, and incubated with 20 mg/ml biotinylated pMHC monomers for 30 min at 4 C. Cells were washed three times after each step. The surfaces of bio-tinylated polystyrene (6 micron diameter, Spherotech) were used as an alternate stimulus of the T-cell. Beads were allowed to react with streptavidine maleimide (Sigma-Aldrich) and incubated with sub saturating concentrations of biotinylated pMHCs for 30min at room temperature.

Adhesion Frequency Assay

This assay uses micromanipulation to precisely set up cell–cell contact with controlled area/time and uses an ultrasensitive force sensor to mechanically detect the presence of adhesion at the end of the contact. The micropipette apparatus uses a pipette to aspirate a T cell and move it in and out of the contact with a pMHC bearing red blood cell. The adhesion sensor in the apparatus is a pipette-aspirated RBC thanks to its ultrasoft membrane that enables the detection of a sub-piconewton force, an order of magnitude smaller than the characteristic force required to break a typical receptor ligand bond, thereby ensuring the detection of single bonds. In the micropipette system, pMHCs are directly coated on the RBC surface and adhesion is detected by visual observation of the RBC membrane deflection. The likelihood of adhesion is estimated from the frequency of adhesions occurred in a large number of contacts made under as close to identical conditions as possible. The present work estimated an adhesion frequency (Pa) from 50 cyclically repeated contacts of a single pair of cells and a mean Pa s.e.m. from several pairs of cells.

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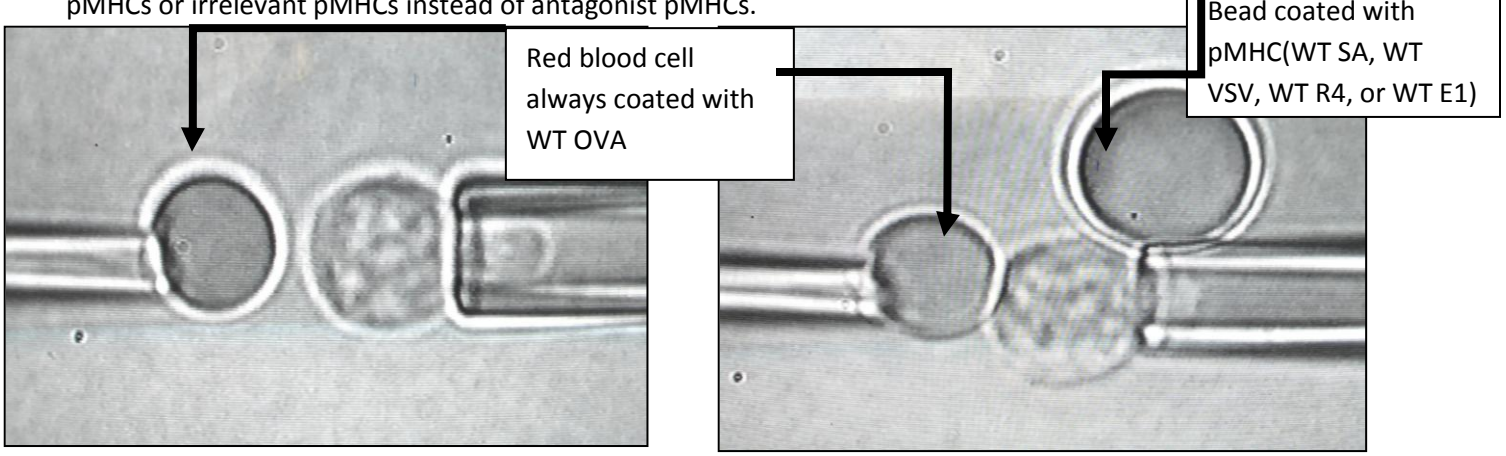
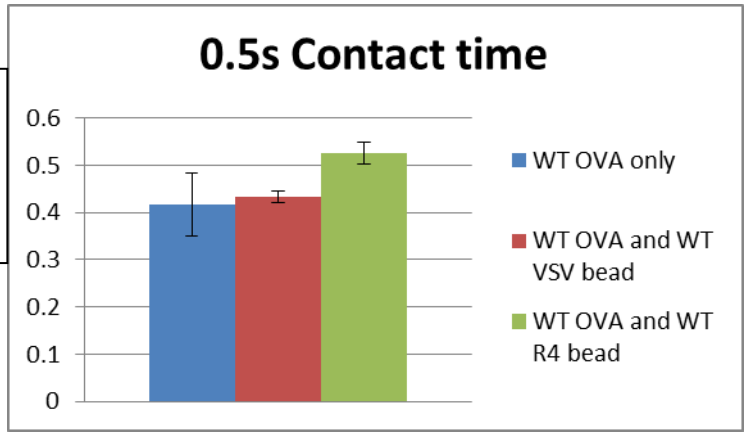
Global T-cell activation Assay Experiment

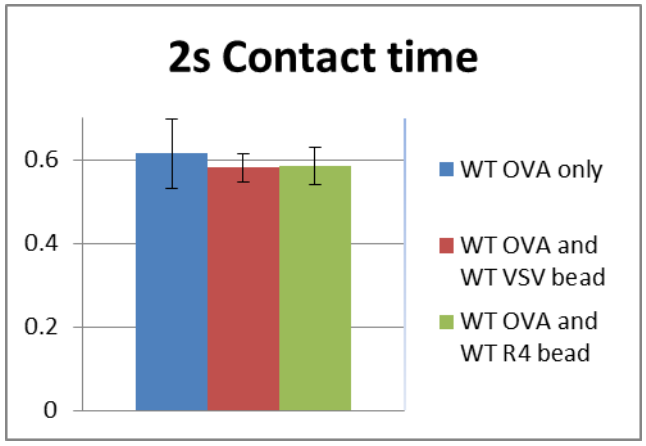
A baseline adhesion frequency using a T cell and a RBC coated with agonist pMHCs was recorded. Then a glass bead coated with antagonist pMHCs was brought into contact with a different spot of the T cell by pressing a microbead on the T-cell, and the adhesion frequency between the RBC bearing agonist pMHC and the T cell was again measured. Control experiments were performed using beads coated with no pMHCs or irrelevant pMHCs instead of antagonist pMHCs.



Graphs: 0.5 and 2s contact times WT OVA(agonist) on red blood cell(positive control), WT VSV(null peptide) on bead(negative control), WT R4 on bead(treatment). WT R4 is an antagonist pMHC.

Average Adhesion Frequency





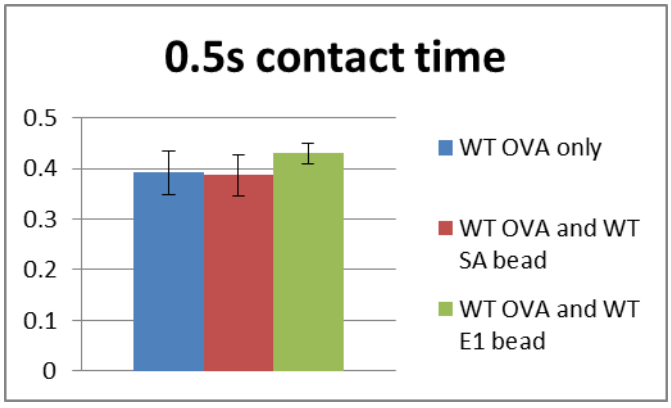
Significantly different mean

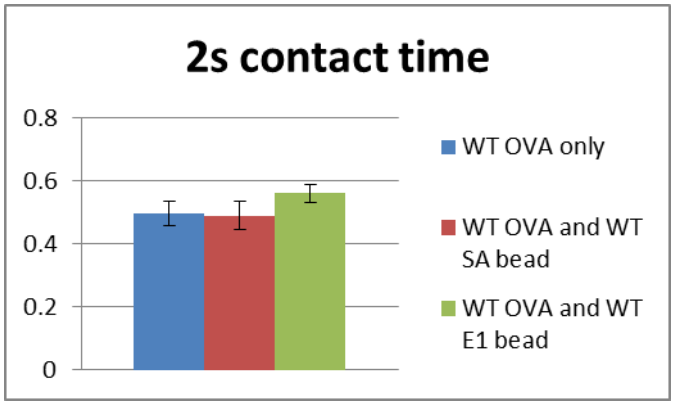
treatment	95% confidence interval	
WT OVA and WT R4 bead	-0.0563	0.0229
WT OVA and WT VSV bead	0.0537	0.1329
WT OVA only	-0.1496	-0.0704

treatment	95% confidence interval	
WT OVA and WT R4 bead	-0.0023	0.0994
WT OVA and WT VSV bead	-0.0329	0.0688
WT OVA only	-0.0202	0.0815

0.5 and 2s contact times WT OVA on red blood cell(positive control), WT SA on bead(negative control), WT E1 on bead(treatment). WT E1 is a weak agonist pMHC.

Average Adhesion Frequency





Significantly different mean

treatment	95% Confidence interval	
WT OVA and WT E1 bead	-0.03	0.0407
WT OVA and WT SA bead	-0.0734	-0.0026
WT OVA only	-0.0787	-0.008

treatment	95% confidence interval	
WT OVA and WT E1 bead	-0.0262	0.0422
WT OVA and WT SA bead	-0.0962	-0.0278
WT OVA only	-0.1042	-0.0358

Statistical Analysis/Results

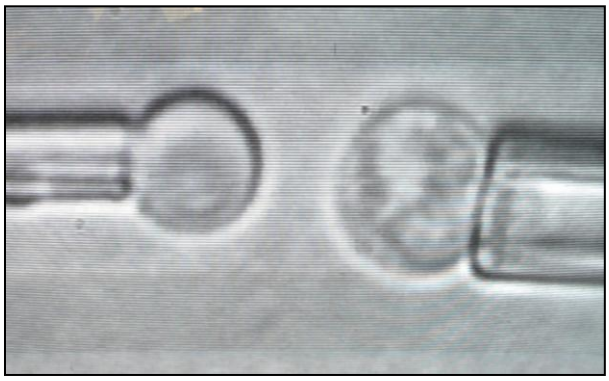
Samples size determined for cell adhesion experiment was based on treatment with largest number of cells used, which varied for each experiment. Graphs error bars represent standard deviation for each average adhesion frequency. Sample size for experiment using WT R4 was twelve (n=12), and for WT E1 experiment was ten (n=10). A one-way ANOVA was used to compare significance between means, and a p-value less than 0.05 ($p<0.05$) was deemed significant. Values from one-way ANOVA were put through a multiple comparison test in MATLAB and means with confidence intervals containing zero were considered significantly different from means whose confidence intervals did not contain zero.

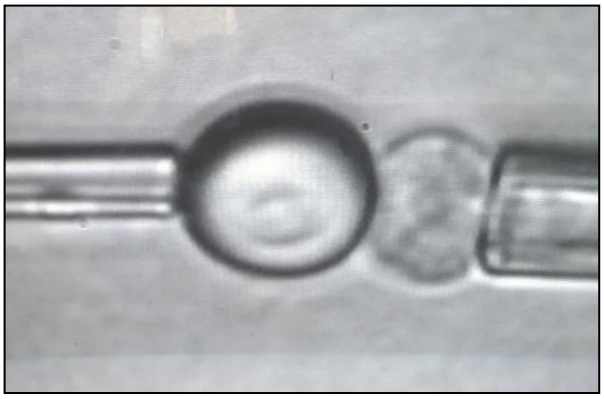
Experiments using WT R4 were analyzed using one-way ANOVA and gave a p value of 1.1420x10^-7 and 0.0748 for 0.5s and 2s contact times respectively. This shows that only the 0.5 second contact time has means that are significantly different. A multiple comparison test confirmed these results with all of the confidence intervals for the 2 second contact time containing zero, and 0.5 second contact time showed the treatment of WT OVA and WT R4 bead was significantly different from both other means.

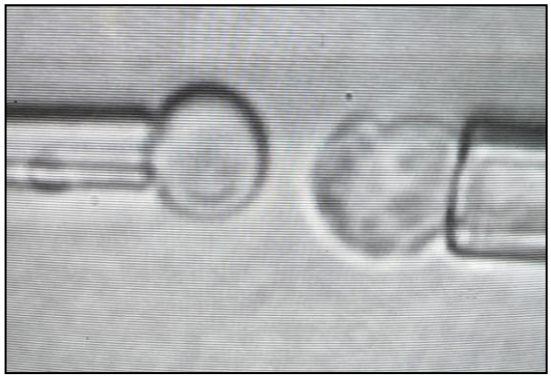
P-values for cell adhesion experiment using WT E1 were 0.01 for 0.5s contact time and 3.47x10^-5 for 2s contact time, showing both graphs had a means significantly different from one another. Upon analysis of results using a multiple comparison test using confidence intervals WT OVA and WT E1 treatment means were significantly different from both WT OVA only and WT OVA + WT SA bead means.

Local time delayed T-cell activation Experiment

This experiment involves a Micropipette Adhesion Frequency assay between a mouse OT1 T-cell and a human Red Blood Cell (RBC) covered with agonist peptide major histocombatibility complex (pMHC) after initial contact with a microbead(6 micrometer diameter) covered with antagonist pMHC for varying time periods. This was done by bringing an antagonist-bearing bead into contact with a T cell for a range of durations and then measuring the change in the effect of modulation of agonist binding.



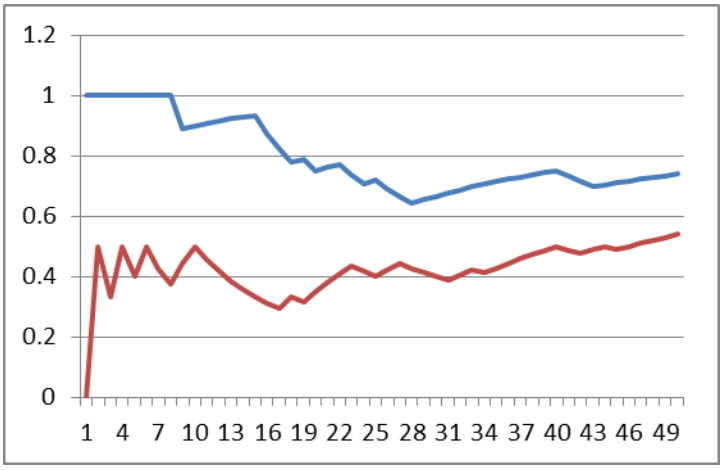


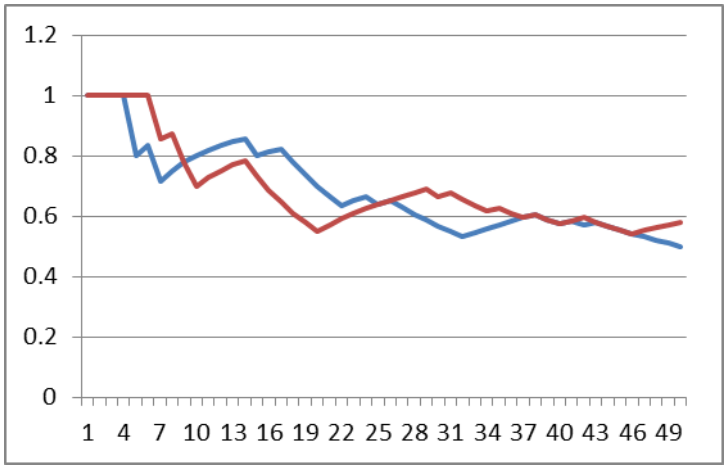


Top left picture is T-cell being stimulated with red blood cell coated with WT OVA, agonist, pMHC. Top right is showing bead coated with WT R4, antagonist, pMHC being held against T-cell for 1 min. Bottom left shows stimulation again with WT OVA pMHC coated red blood cell.

Blue = running frequency of T-cell before antagonist stimulation with bead

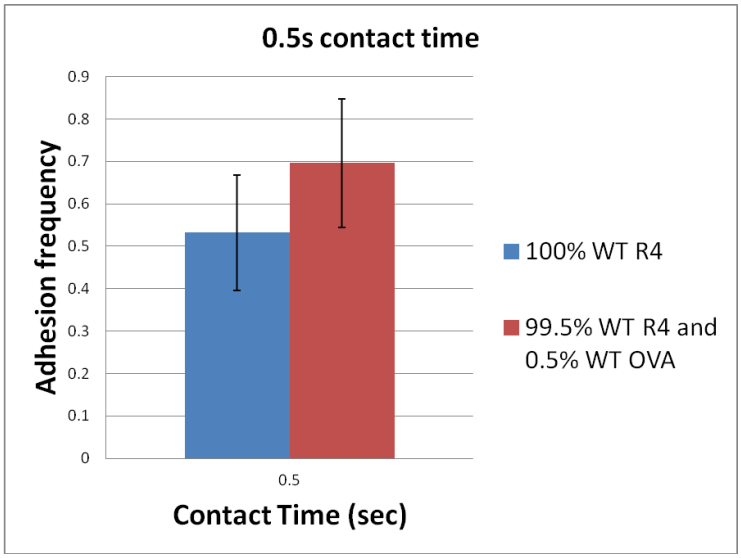
Red = running frequency of T-cell after antagonist stimulation with bead for 1 min

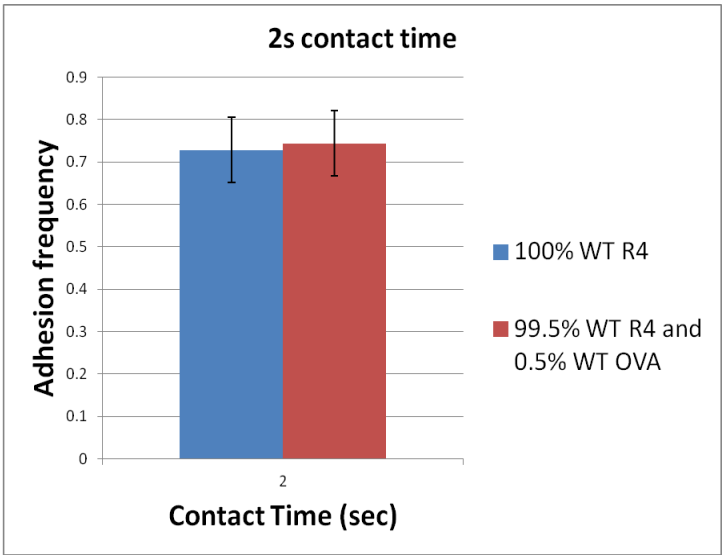




Local w/o time delayed T-cell activation (simultaneous stimulation)

This is extremely similar to the Local time delayed T-cell activation experiment; however, this experiment has no microbead involved. Instead the RBC is covered with mixture of antagonist and agonist pMHC to allow us to see what will happen when the agonist and antagonist are presented not only locally, but also simultaneously.





Discussion

Experiment	Agonist Peptide Used	Antagonist Peptide Used	Adhesion Frequency(0.5 seconds, 2 seconds)
Global stimulation	WT OVA	WT E1 and WT R4	WT E1 (increase, increase) WT R4 (increase, no change)
Local Time-Delayed stimulation	WT OVA	WT R4	Results inconclusive
Local w/ Simultaneous stimulation	WT OVA	WT R4	Increase, no change

Although micropipette adhesion frequency assays have only been used for the past 15 years (Chesla et al. 1998), trends seen from these experiments can point how to interpret certain interactions between cells that have never been quantified. For instance in the global stimulation the antagonist coated bead was not always placed the same distance from the agonist contact point, nor was it in the same spot for different cells; however, by using this stimulation protocol it is now possible for us to understand how these antagonist ligands seem to not distract the cell from agonist stimulation, but instead enhances this interaction. This is something that could not have been inferred from previous experimentation on T-cells, nor could have been observed if adhesion assays had not been used.

Another interesting observation from these experiments was the interaction between agonist and antagonist ligands when both were presented to the T cell in the same place and time. This showed that even when agonist ligand was present in a small amount compared to antagonist ligand the T cell showed a large increase in the frequency of the cell recognizing this ligand. Although this result was expected since affinity for agonist is much higher than that of antagonist, this makes understanding the activation of T cells even easier due to the fact that a similar response was shown in the global stimulation. However in global stimulation experiments, it was in a way that shows how antagonist ligands seem to repel the attention of the cell away from antagonist ligand and bring the cells' attention more towards agonist. Since contact times below one second showed similar reactions of increased adhesion frequency between these two stimulations that could prove that the road towards activation could be decided within the first minute of stimulation. This proves how quickly and precisely these cells work in recognizing antigen within the body.

Conclusion

Overall these experiments showed how fast recognition of an antigen by T-cells and subsequent T cell activation might be *in situ*. Now that the time scale and type of stimulation has been narrowed down we can move forward in a more thorough manner to investigate how these stimulations cause the first step of T-cell activation. Understanding this could help immunologist study why certain diseases or injuries cause the type of responses within the body that can seem perplexing. Furthermore how these cells activate could give insight into why in some cases T-cells become activated and begin attacking healthy tissues in the body like in the case of autoimmune diseases like multiple sclerosis and diabetes type 1. Knowing the mechanism of T-cell activation is a fundamental part of our immune response and once we know how it works could give us greater understanding of the immune system as a whole.

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